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Genetic diversity of *Escherichia coli* isolates in irrigation water and associated sediments: implications for source tracking

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Abstract

Identifying the sources of fecal contaminants in surface water bodies such as rivers and lakes is of significant importance for environmental quality, food safety and regulatory purposes. Current DNA library-based source tracking approaches rely on the comparison of the genetic relatedness among the fecal contaminants. The objective of this study was to determine the genetic relatedness of *Escherichia coli* isolated from irrigation water and associated sediments using pulse field gel electrophoresis (PFGE) and to evaluate the genetic stability of the *E. coli* PFGE patterns. The isolates were obtained over a 4-month period from specific locations within irrigation canals and sediments associated with the Rio Grande River along the Texas–Mexico border. Fifty *E. coli* isolates were genotyped using PFGE. Different *E. coli* genotypes were identified among samples collected in 11 different locations. Some isolates obtained over successive months showed similar genotypic patterns. In the laboratory experiment, the PFGE pattern of one *E. coli* strain changed during survival in irrigation water. The genetic relatedness of this strain changed from >95% to <83% over 8-week survival. These results imply that PFGE is of such extreme resolution that it may be a challenging task to rely solely on a PFGE-based source tracking DNA fingerprint library for large watersheds.

Keywords: Pulse field gel electrophoresis; *Escherichia coli*; Source tracking; Irrigation water; Genetic diversity

1. Introduction

Fecal contamination of water resources is a major issue that confronts a number of countries. In the United States, the total maximum daily load (TMDL) regulations of the US Environmental Protection Agency (USEPA) require state and local water agencies to identify the various inputs, quantify their respective contributions to the impairments of water bodies and develop appropriate remediation strategies (USEPA, 2002). Thus it is critical that a clear understanding of the probable sources of fecal contaminants be achieved (Bernstein et al., 2002). Different approaches have been proposed to help in identifying the probable contamination sources (Bernstein et al., 2002). The methods can be broadly divided into library-dependent and library-independent methods (Carson et al., 2001; Dombek et al., 2000; Guan et al., 2002; Harwood et al., 2000; Hsu et al., 1995; Samadpour, 2002). DNA fingerprinting-based methods such as ribotyping, pulse field gel electrophoresis (PFGE) and rep-PCR have been reported as candidate library-dependent methods (Carson

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et al., 2001; Dombek et al., 2000; Krause et al., 1996; Parveen et al., 2001).

Irrigation water, which can be a source of pathogenic microorganisms, can ultimately contaminate agricultural products (Beuchat and Ryu, 1997; Guo et al., 2001; Solomon et al., 2002; Thurston-Enriquez et al., 2002). A variety of fecal contaminants and pathogens such as *E. coli*, *Salmonella* spp., *Listeria* spp., *Vibrio cholerae*, *Crytosporidium*, and enteric viruses have been isolated from irrigation water and associated sediments (Borchardt and Spencer, 2002; Jiang et al., 2000; Lu et al., 2002; Morace et al., 2002). Given the importance of food safety to modern society, the identification of the possible sources of fecal contamination in these types of waters can make a significant impact on the production of microbiologically safe fruits and vegetables, and in the development of appropriate management strategies (Beuchat and Ryu, 1997; Proctor et al., 2001).

A key prerequisite for DNA fingerprinting-based source identification is an understanding of the genetic relatedness of the bacterial isolates. PFGE is considered an extremely powerful sub-typing molecular method. Differentiation of microorganisms is based on DNA sequence-level variations among the genomes observed after site-specific restriction endonuclease digestion of chromosome DNA (Barrett et al., 1994). PFGE-based fingerprinting currently serves as the gold standard for epidemiological tracking of organisms by the Center for Disease Control and Prevention (Swaminthan et al., 2001). The procedure has been successfully used for the investigation of infectious sources, and genetic diversity of pathogens (Baggesen et al., 2000; Kariuki et al., 1999; Swaminthan et al., 2001). In order to successfully track the origin (human or animal) of fecal bacteria using DNA fingerprinting methods, it is critical that the genetic profile of the isolates be stable and that the DNA fingerprint pattern can be linked to a particular host (Samadpour, 2002).

The objective of the present study was to determine the genetic relatedness of *E. coli* isolates obtained from irrigation water and associated sediments using PFGE. The underlying hypothesis was that *E. coli* isolates can exhibit significant genetic diversity in a natural environment, and that their PFGE patterns can change during their survival in such environments complicating source tracking. We tested the hypothesis using *E. coli* isolates from a large stretch of the Rio Grande River along the Texas–Mexico border.

2. Materials and methods

2.1. Sample collection

Water and sediment samples were collected from irrigation canals at 11 locations along the Texas–Mexico border (Fig. 1). The samples were collected on a monthly basis for four months (June 2000–September, 2000) using standard microbiological sampling protocols (American Public Health Association, 1998). Water samples were collected in sterile plastic bottles while sediment samples were collected using surface sterilized scoops and placed in sterile plastic bags. The samples were shipped to the laboratory on Blue Ice™ and maintained at 4 °C until analysis. The samples were analyzed for *E. coli* using standard methods as detailed below (American Public Health Association, 1998).

2.2. E. coli isolation

Twenty grams (wet weight) sediment samples were added to 200 ml of 1% peptone and thoroughly mixed. Portions were removed and enriched overnight in EC broth (Acumedia, Baltimore, MD) at 44.5 °C and subsequent plating on MacConkey agar (Acumedia, Baltimore, MD). The *E. coli* presumptive colonies were confirmed using LB-MUG fluorescence (Acumedia, Baltimore, MD) (Tryland and Fiksdal, 1998; Venkateswaran et al., 1996). One hundred milliliters of each water sample was filtered through a sterile 0.45 μm membrane (Gelman Sciences, Ann Arbor, MI), and incubated on m-Coliblue 24® media (Hach, Loveland, CO) at 37 °C for 24 h. The blue-colored colonies were confirmed as *E. coli* using LB-MUG fluorescence and isolates stored in 25% glycerol at −20 °C prior to PFGE analysis. Table 1 is a list of *E. coli* isolates and their sources.

2.3. Pulse field gel electrophoresis

PFGE was performed as described by Barrett et al. (1994) with minor modifications. Overnight cultures (37 °C) of *E. coli* in Trypticase Soy Broth (Difco, Detroit, MI) were harvested by centrifugation at 20 °C for 7 min at 8000*g*. The cells were washed twice with phosphate buffered saline (PBS, 0.01 M, pH 7.2), and suspended in PBS to adjust cell concentrations to approximately 10^8–10^9 CFU/ml using OD_{600} measurements. Equal volumes of pre-heated (45 °C) 1% low melting point agarose (Bio-Rad Laboratories; Richmond, CA) and cell suspensions were mixed gently. The mixtures were poured into molds and held at 4 °C to solidify plugs. Plugs were incubated overnight at 50 °C in lysis solution (0.5 M EDTA (Sigma Chemical Company, St. Louis, MO), pH 9.3, 1% *N*-lauroylsarcosine (Sigma Chemical Company), 0.2 mg/ml Proteinase K (Boehringer Mannheim; Indianapolis, IN)). One plug from each sample was treated with phenylmethylsulfonyl fluoride (Sigma Chemical Company), and washed for 30 min three times with TE (10 mM Tris (USB Specialty Biochemicals, Division of Amersham Life Science, Inc.; Cleveland, OH), pH 8.0; 1 mM EDTA) followed by equilibration with digestion buffer

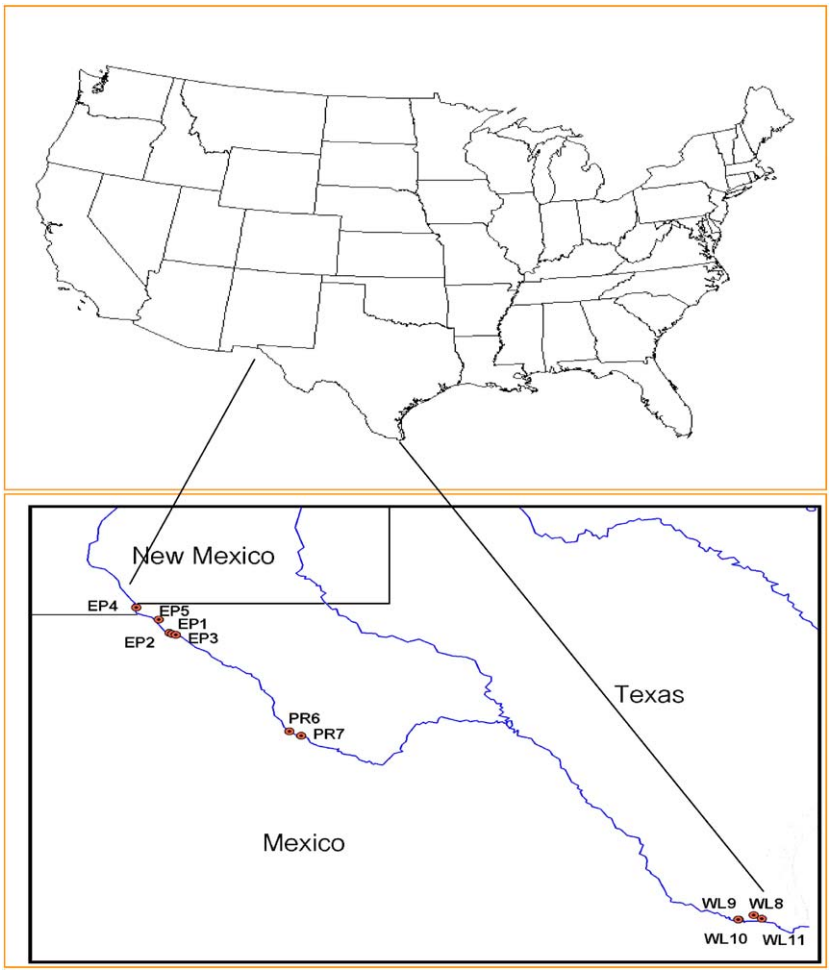

Fig. 1. Sampling locations along Texas–Mexico border (EP = El Paso; PR = Presidio and WL = Weslaco).

for *Xba*I according to manufacturer's instruction (New England BioLabs, Beverly, MA). One quarter of each plug was digested overnight with 20U of *Xba*I at 37 °C and was placed in wells of a 1% agarose gel (Roche Diagnostics, Indianapolis, IN). PFGE was performed on a CHEF Mapper® XA Pulsed Field Gel Electrophoresis System (Bio-Rad) in 0.5 × TBE (Tris-Broic acid-EDTA (0.089 M Tris, 0.089 M boric acid, 0.001 M EDTA (pH 8.0)) for 22 h at 12 °C with initial switch time of 1 s, final switch time of 90 s, and voltage of 6.0 V/cm. Gels were stained with ethidium bromide for 30 min and de-stained with 1 mM of magnesium sulfate for another 30 min. Images were recorded using a digital imaging system (ChemiImager 4000 Low Light Imaging System, AlphaEase Software, version 3.3a, Alpha Innotech Corporation, San Leandro, CA).

Dendrograms were created using Molecular Analyst® Fingerprinting software (Version 1.6, Bio-Rad Hercules, CA). All parameters were set as default mode. A molecular marker (lambda ladder PFG marker, New England BioLabs) was chosen as standard reference to normalize PFGE patterns for comparison of different gels. Clusters were created on basis of similarity coefficient calculated using UPGMA method. Similarity coefficient ranged from 0% to 100%, corresponding to the portion of shared fragment patterns. When the coefficient was greater than 90% (cut-off value), isolates were considered to have the same PFGE pattern and genetically similar (Tenover et al., 1995).

2.4. Stability of PFGE patterns during *E. coli* survival in filtered irrigation water

Three *E. coli* isolates (EP4H603, WL8S666 and WL10S977) obtained from El Paso and Weslaco locations and isolated from irrigation water and sediment samples were employed in these studies. The glycerol pure cultures were grown overnight (18 h) at

Table 1
E. coli isolates analyzed by Pulse Field Gel Electrophoresis

Strain	Species	Location	Source	Date of isolation
EP4H603	*E. coli*	EP4	Water	June 2000
EP4H605	*E. coli*	EP4	Water	June 2000
EP4H606	*E. coli*	EP4	Water	June 2000
EP4H672	*E. coli*	EP4	Water	June 2000
EP4H704	*E. coli*	EP4	Water	July 2000
EP4H756	*E. coli*	EP4	Water	July 2000
EP4H764	*E. coli*	EP4	Water	July 2000
PR6H609	*E. coli*	PR6	Water	June 2000
PR6H610	*E. coli*	PR6	Water	June 2000
PR6S611	*E. coli*	PR6	Sediment	June 2000
PR6S612	*E. coli*	PR6	Sediment	June 2000
PR6S687	*E. coli*	PR6	Sediment	June, 2000
PR7S660	*E. coli*	PR7	Sediment	June 2000
PR7S668	*E. coli*	PR7	Sediment	June 2000
PR7S788	*E. coli*	PR7	Sediment	July 2000
PR7H675	*E. coli*	PR7	Water	June 2000
PR7H752	*E. coli*	PR7	Water	July 2000
PR7H976	*E. coli*	PR7	Water	September 2000
PR7H983	*E. coli*	PR7	Water	September 2000
WL8H680	*E. coli*	WL8	Water	June 2000
WL8S666	*E. coli*	WL8	Sediment	June 2000
WL8S758	*E. coli*	WL8	Sediment	July 2000
WL8S773	*E. coli*	WL8	Sediment	July 2000
WL8S953	*E. coli*	WL8	Sediment	September 2000
WL8S961	*E. coli*	WL8	Sediment	September 2000
WL8S969	*E. coli*	WL8	Sediment	September 2000
WL8S985	*E. coli*	WL8	Sediment	September 2000
WL9S648	*E. coli*	WL9	Sediment	June 2000
WL9S791	*E. coli*	WL9	Sediment	July 2000
WL9S793	*E. coli*	WL9	Sediment	July 2000
WL9S949	*E. coli*	WL9	Sediment	September 2000
WL9S957	*E. coli*	WL9	Sediment	September 2000
WL9S965	*E. coli*	WL9	Sediment	September 2000
WL10H650	*E. coli*	WL10	Water	June 2000
WL10S681	*E. coli*	WL10	Sediment	June 2000
WL10S690	*E. coli*	WL10	Sediment	June 2000
WL10S774	*E. coli*	WL10	Sediment	July 2000
WL10S859	*E. coli*	WL10	Sediment	August 2000
WL10S867	*E. coli*	WL10	Sediment	August 2000
WL10S870	*E. coli*	WL10	Sediment	August 2000
WL10S989	*E. coli*	WL10	Sediment	September 2000
WL10S951	*E. coli*	WL10	Sediment	September 2000
WL10S962	*E. coli*	WL10	Sediment	September 2000
WL10S977	*E. coli*	WL10	Sediment	September 2000
WL11H601	*E. coli*	WL11	Water	June 2000
WL11H602	*E. coli*	WL11	Water	June 2000
WL11S608	*E. coli*	WL11	Sediment	June 2000
WL11H654	*E. coli*	WL11	Water	June 2000
WL11H678	*E. coli*	WL11	Water	June 2000
WL11H686	*E. coli*	WL11	Water	June 2000

37 °C on TSA plates. A single isolated colony of each isolate was picked and cultured overnight at 37 °C in Tryptic Soy Broth. The cells were harvested by centrifugation at 8000*g* for 10 min at room temperature and washed three times by repeated centrifugation and suspension in sterilized de-ionized water. Cell

concentrations of each strain were adjusted to 10^9 CFU/ml prior to inoculation into separate sterile flat-bottom flasks containing 150 ml of 0.45 μm filtered irrigation water sample previously obtained from the Weslaco region. The flasks were wrapped in aluminum foil and maintained without shaking at room temperature (25 °C) to simulate a puddle within the irrigation canal.

Three replicate samples (1 ml) were removed from each flask on a weekly basis for two months. The samples were serially diluted in 0.1% peptone and plated on R2A plates and enumerated after an overnight incubation at 37 °C. The picked colonies were confirmed as being *E. coli*. At each sampling, six isolates of each of three strains were arbitrarily selected for PFGE analysis. The resulting PFGE patterns were analyzed as described earlier.

3. Results

3.1. Genetic diversity of E. coli isolates

Fifty *E. coli* isolates obtained from 11 different locations along the Texas–Mexico border from water and sediment samples were genotyped by PFGE (Table 1, Figs. 2A, B and C). Isolates obtained from the same location over a period of 4 months were analyzed together to understand their genetic diversity. Twelve isolates were collected at Presidio (6 water and 6 sediment isolates), 7 isolates from El Paso (all water isolates), and 31 isolates from the Weslaco region (6 water and 24 sediment isolates). Eleven different genotypes were identified among the 12 *E. coli* isolates from samples collected at Presidio (Fig. 2A). Other than the 2 isolates (PR6S687 and PR6S611) that were collected at the same site during the same sampling showed almost 100% genetic similarity, all of the other isolates exhibited <90% genetic relatedness. Isolates obtained from the sediment in the same month (PR7S668 and PR7S660) at the same site were genetically diverse based on the 90% cut-off value. The enrichment method used to isolate the *E. coli* strains could have biased the diversity to certain genotypes. Four different genotypes were identified among the seven *E. coli* strains from El Paso water samples (Fig. 2B). Isolates collected in the same month (e.g., EP4H672 and EP4H605 in June, EP4H756 and EP4H764 in July) from same sample type were genetically diverse. Interestingly, three *E. coli* isolates (EP4H603, EP4H704, and EP4H764) collected in different months from water sample had identical patterns. The Weslaco isolates were grouped into 25 genotypes (Fig. 2C). Isolates, such as WL9S965 and WL9S957, collected in September from sediment samples at WL9, WL8S758 and WL8S773 collected in July from sediment samples at site WL8, were genetically diverse. Two isolates, WL10S870 and WL10S951 from sediment samples in the successive months (August and September) at site WL10 had the identical patterns. Isolates such as WL11H601 and WL11H686, WL10S681 and WL10S690, and WL11S608 and WL11H654, with the same patterns and collected in the same month at the same site were considered genetically similar.

3.2. Stability of PFGE patterns during E. coli survival in filtered irrigation water

The stability of the PFGE patterns of three *E. coli* isolates (EP4H603, WL8S666 and WL10S977) that were originally isolated from water and sediment samples were studied under laboratory conditions (Fig. 3A). Six *E. coli* isolates of each sample taken in each week were selected for PFGE genotyping. No change was found in PFGE patterns for EP4H603 and WL8S666 in the duration of 8 weeks. However, a new PFGE pattern was observed for one clone of the isolate WL10S977 at the eighth week. This particular clone showed a significant shift in the 533.5 kb fragment range (Fig. 3A, lane 15), which was different from the other strains obtained in the study. Dendogram analysis (Fig. 3B) indicated that the genetic relatedness of this particular clone was only 82.6% as compared to the other five clones of the same isolate.

The survival of three *E. coli* isolates (EP4H603, WL8S666 and WL10S977) based on R2A plate counts is shown in Fig. 4. All three strains survived for eight weeks in the filtered irrigation water. The three isolates, however, showed differences in their survival patterns. Isolates WL8S666 and WL10S977 showed an inactivation rate of 0.4 log/ml/day, while isolate EP4H603 exhibited an inactivation rate of 0.9 log/ml/day. At the end of 8 weeks, the number of surviving cells of WL8S666 was significantly higher ($p<0.05$) than the other two isolates. However, the number of viable *E. coli* cells of three experimental isolates ranged between 10^3 and 10^6 CFU/ml.

4. Discussion

The results document the presence of fecal indicator bacteria in the irrigation water along the Texas–Mexico border. The PFGE results of this study indicate considerable genetic diversity among *E. coli* isolates from water and sediment samples even in one location collected over a four-month period from irrigation canals along the Rio Grande. Multiple isolates obtained from the same sample location, the same sample type and sampling time frame showed genetic diversity. There are possible explanations behind these wide patterns of diversity. Under a simplistic scenario, the isolates could

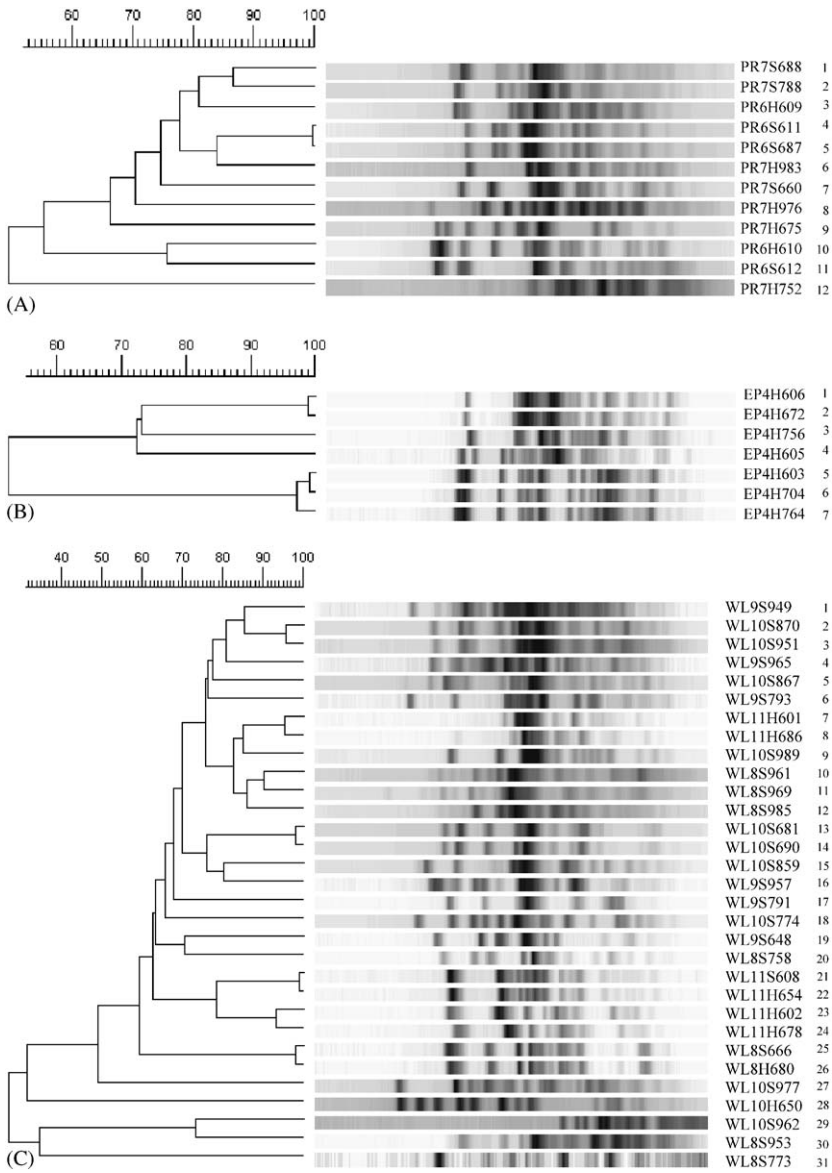

Fig. 2. PFGE pattern and dendrogram analysis of *E. coli* isolates obtained from irrigation water and associated sediments collected at (A) Presidio, water isolates (lane 3, 6, 8–10, 12) and sediments isolates (lane 1, 2, 4, 5, 7, 11). June isolates (lane 3–5, 10, 11), July isolates (lane 1, 2, 7, 9, 12) and September isolates (lane 6, 8); (B) EP4 site, water isolates. June isolates (lane 1, 2, 4, 5) and July isolates (lane 3, 6, 7); (C) Weslaco, water isolates (lane 7, 8, 22, 23, 24, 26, 28) and sediments isolates (lane 1 to 6, 9–21, 25, 27, 29–31). June isolates (lane 7, 8, 13, 14, 19, 21–26, 28), July isolates (lane 6, 17, 18, 20, 31), August isolates (lane 2, 5, 15) and September isolates (lane 1, 3, 4, 9–12, 16, 27, 29, 30).

have originated from entirely different sources. But significant genotype diversity was found even within a subset of *E. coli* (Hancock et al., 1998; Krause et al., 1996). Alternatively, genomic rearrangement during the survival and persistence of these enteric bacteria is a possibility (Finkel et al., 2000; Nass et al., 1994; Riehle et al., 2001).

Interestingly, three *E. coli* isolates (EP4H603, EP4H704 and EP4H764) in EP4 site and two isolates (WL10S870 and WL10S951) in WL10 site collected on the successive months had identical patterns. Since enteric bacteria such as *E. coli* and *Salmonella* can survive and multiply in aquatic environments especially in subtropical environments (Baudart et al., 2000;

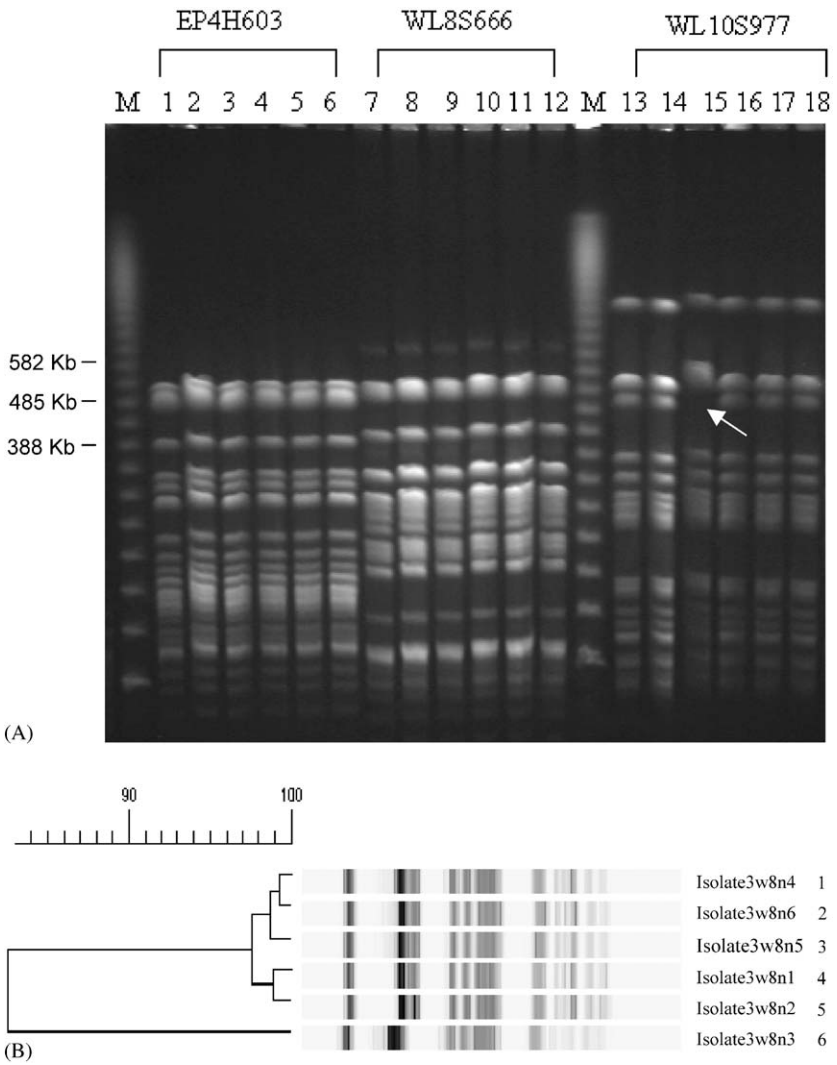

Fig. 3. Stability of *E. coli* PFGE pattern in laboratory microcosm after 8 weeks. (A) PFGE patterns of the three strains at 8 weeks post-inoculation in filtered irrigation water at ambient temperature. Lanes M, Lambda DNA ladder PFG marker. Lanes 1–6 are clones of EP4H603 isolate. Lanes 7–12 are clones of WL8S666 isolate. Lanes 13–18 are clones of WL10S977 isolate. Arrow indicates the difference in the banding pattern. (B) Dendrogram of PFGE patterns for WL10S977 isolates at 8 weeks post-inoculation.

Desmarais et al., 2002; Maule, 2000; Samadpour, 2002), we did the survival and genotype persistence experiments of *E. coli* under starvation conditions in the laboratory. Results from this study show that *E. coli* when exposed to irrigation water in the absence of biotic influence can persist for up to 8 weeks (Fig. 4), and even at the end of 8 weeks the numbers of viable *E. coli* isolate (which exhibited PFGE variability) averaged approximately 10^4 CFU/ml for an original starting concentration of 10^8 CFU/ml. The declining numbers of *E. coli* during these 8 weeks might be the net difference between cell death and possible multiplication. The differences in survival among the three *E. coli* strains suggest that certain genotypes may have unique survival patterns. The presence of certain genotypes in samples collected for source tracking studies may be related to the different survival patterns and should be taken into consideration when interpreting the results.

These results are in agreement with other reports that have demonstrated *E. coli* can survive and multiply in irrigation water, wastewater, subtropical sediments, and mineral water (Baudart et al., 2000; Davies et al., 1995; LaLiberte and Grimes, 1982; LeJeune et al., 2001; Ramalho et al., 2001; Solo-Gabriele et al., 2000). Additionally, the irrigation water sample and the isolate

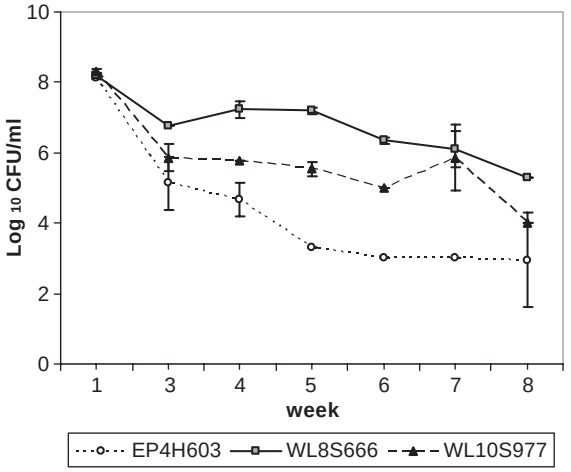

Fig. 4. Survival of *E. coli* isolates in laboratory microcosm. Each data point represents the mean of three replicate flasks and the error bars denote standard deviation.

WL8S666 that was employed in the survival study were isolated from Weslaco, which is in a subtropical region of Texas (Texas Nautral Resouce Conservation Commission, 1998; Texas Water Commission, 1990). Cell debris such as amino acid molecules released by dead cells can be taken up for cell growth and maintenance during starvation conditions (Finkel et al., 2000). Nutrients in the irrigation water supply can support *E. coli* multiplication. Previous studies have reported that *E. coli* can proliferate in subtropical ecosystem (Solo-Gabriele et al., 2000; Toranzos, 1991; Wright, 1989).

The alternation of PFGE banding patterns during prolonged survival in one of three clones could have arisen for the loss of a restriction site on the genome, which might be caused by mutation or recombination and confer expression of growth advantage in stationary phase (GASP) phenotype (Finkel et al., 2000; Zambrano et al., 1993). Mutation rate in *E. coli* increases during prolonged survival, and that is due to decrease in replication fidelity (Finkel and Kolter, 1999, 2001). Studies have demonstrated that DNA fragments from dead cells can become integrated into viable cells by homology-facilitated illegitimate recombination (de Vries and Wackernagel, 2002; Prudhomme et al., 2002). This can further alter PFGE patterns.

These results showed that there is significant *E. coli* diversity in irrigation water and associated sediments. Additionally, the alternation of PFGE patterns in *E. coli* during extended survival in irrigation water raises the question regarding the applicability of sensitive fingerprinting techniques such as PFGE for source typing. DNA library-based source typing methods currently rely on manual or automated ribotyping, rep-PCR, and PFGE analysis (Bernstein et al., 2002; de Cesare et al., 2001; Hagedorn, 2002; Hartel, 2002; Olive and Bean, 1999). Automated ribotyping is not sensitive enough to discern genetic diversity for epidemiological purposes. Manual ribotyping and rep-PCR have not been standardized thus far and so, and they are prone to laboratory-based variations. Under these circumstances PFGE, since it has been standardized and used extensively, will continue to be the standard for discerning genetic relatedness among isolates. The results of this study show that even a minor change in the banding pattern can significantly affect clusters. Thus, to employ PFGE for source tracking in watersheds like the Rio Grande River, a very extensive PFGE fingerprint library is needed. The DNA fingerprint library has to be comprehensive enough to account for the potential multiple contamination sources and accommodate the spatial and temporal genetic variability of *E. coli* strains. The library should also take into account the possible genetic diversity fluctuations that can occur within strains during survival in such environments.

5. Conclusion

Overall, these results imply that accurate source identification of fecal contaminants in large watersheds can be an extremely challenging task given the current state of the science. There needs to be a concerted attempt at side-by-side comparison of DNA fingerprinting methods such as ribotyping and PFGE using a large number of isolates and subsequent field-testing of the appropriate methods. Such an approach will help to identify the method(s) that can discriminate between different host-specific lineages without being overly sensitive. Alternatively, library independent methods such as genotyping F^+ RNA coliphages and detection of human fecal sterols are methods that need to be further developed (Schaper et al., 2002). The development and refinement of such approaches are urgently needed so that they can be applied to large watersheds (such as the one example in this study) where multiple contamination sources exist, where fecal contaminants can persist for extended periods of time, and genomes can undergo genetic rearrangements.

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